Polymerase synthesis of four-base DNA from two stable dimeric nucleotides

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ABSTRACT

We document the preparation and properties of dimerized pentaphosphate-bridged deoxynucleotides (dicaptides) that contain reactive components of two different nucleotides simultaneously. Importantly, dicaptides are found to be considerably more stable to hydrolysis than standard dNTPs. Steady-state kinetics studies show that the dimers exhibit reasonably good efficiency with the Klenow fragment of DNA polymerase I, and we identify thermostable enzymes that process them efficiently at high temperature. Experiments show that the dAp_5dT dimer successfully acts as a combination of dATP and dTTP in primer extension reactions, and the dGp_5dC dimer as a combination of dGTP and dCTP. The two dimers in combination promote successful 4-base primer extension. The final byproduct of the reaction, triphosphate, is shown to be less inhibitory to primer extension than pyrophosphate, the canonical byproduct. Finally, we document PCR amplification of DNA with two dimeric nucleotides, and show that the dimers can promote amplification under extended conditions when PCR with normal dNTPs fails. These dimeric nucleotides represent a novel and simple approach for increasing stability of nucleotides and avoiding inhibition from pyrophosphate.

INTRODUCTION

DNA polymerases are universally important in research laboratories and in biomedical applications. They are extensively employed in nucleic acid amplification methodologies such as polymerase chain reaction (1) (PCR) and rolling circle amplification (2), as well as in sequencing (3) and structure mapping (4). Techniques relying on polymerase primer extension are essential to molecular biology and genomics research, clinical diagnosis, forensics and a variety of other applications (5–7). Sequencing and structure mapping are increasingly useful techniques that allow researchers to ac-

quire biologically important information about specific nucleic acids (8). Simple alterations to these methodologies that address some of their limitations may allow for increased progress and productivity across many fields and applications.

The canonical substrates that polymerases use to synthesize DNA are deoxynucleoside triphosphates (dNTPs), which consist of a nucleobase bound to deoxyribose, with a chain of three phosphate residues at the 5′-hydroxyl on the sugar. Naturally occurring as derivatives of each of the four common nucleobases, the canonical dNTPs are deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), and deoxycytidine triphosphate (dCTP). DNA polymerases use dNTPs to extend a DNA primer hybridized to a complementary DNA template. As the appropriate dNTP is incorporated into the growing strand, a phosphodiester bond is formed between the 3′-hydroxyl terminus on the growing strand and the α-phosphate of the incoming dNTP (9). Pyrophosphate is released as the byproduct of this reaction (9).

Several issues with dNTPs place limits on their utility in application. dNTPs are prone to hydrolysis, yielding deoxynucleoside diphosphate (dNDP) and orthophosphate under aqueous conditions (10). Exposure to elevated temperatures, which is commonly employed for ameliorating issues of inhibitory secondary structure as well as during thermal cycling, further promotes this decomposition. This is also an issue with long-term storage of dNTPs, leading to a commonly observed failure of PCR when older stocks of dNTPs are used (11). Furthermore, the byproduct of DNA synthesis, pyrophosphate, can also act to inhibit primer extension when its concentration builds over time. This phenomenon, termed pyrophosphorolysis, entails the enzyme-catalyzed chemical reverse of the primer extension reaction in which the primer strand is attacked by pyrophosphate, shortening the primer by iterative excision of terminal nucleotides, and regenerating dNTPs in the process (12). Pyrophosphorolysis becomes especially problematic with long range PCR involving large genomic templates (>20 kb) or PCR performed on extremely dilute samples, since longer

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extension times and extended cycle counts contribute to pyrophosphate buildup (13,14).

One possible solution to the pyrophosphorolysis problem is the use of deoxynucleoside tetra- or penta-phosphates, which would yield triphosphate or tetraphosphate as byproducts rather than pyrophosphate (15). However, these do not have increased hydrolytic stability relative to dNTPs, and their use entails synthesis of four modified nucleotides. Deoxynucleoside diphosphates have also been shown to function with select polymerases, although these are relatively poor substrates and suffer from the same shortcomings as their canonical analogs (16). As for the stability issue, one study described increased thermal stability of nucleoside triphosphates with alkyl groups appended to the terminal phosphate (17). While thermal stability is a benefit, attaching these types of linkers could impede polymerase reaction rates while the alkyl group itself does not contribute to the polymerase reaction itself. This approach also necessitates the synthesis of four modified nucleotides, an involved and tedious process.

Here we report a novel strategy aimed at addressing these concerns. We have designed two distinct chimeric nucleotide dimers each containing a pentaphosphate bridge (Figure 1A). Analogous to nucleoside polyphosphates with appended alkyl groups (17), these dimers lack a terminal phosphate exposed to water, so they are potentially less labile under aqueous conditions relative to nucleoside polyphosphates. We envisioned that these two-headed nucleotides, or 'dicaptides' (from the Latin *capita* for 'heads'), could potentially be suitable substrates for polymerases. Surprisingly, such pentaphosphate deoxynucleotide dimers have not yet been reported to our knowledge. Our previous studies with *tetra*phosphate-bridged deoxy/ribo chimeric nucleotides have shown good DNA polymerase activity with the deoxynucleotide end of the molecules (18,19), establishing that an extra nucleotide outside the active site does not necessarily interfere with polymerase activity. In principle, after a dicaptide is used by polymerase to extend a primer strand by one nucleotide, the leaving group is a nucleoside tetraphosphate (Figure 1B). Nucleoside tetraphosphates have been shown to be excellent substrates for polymerases (15). After incorporation of the remaining nucleoside tetraphosphate, the final byproduct is triphosphate, which we hypothesized might be less inhibitory toward polymerase primer extension than pyrophosphate. Additionally, whereas native dNTPs yield one equivalent of pyrophosphate for each single-base extension, dicaptides would produce only one triphosphate molecule per two nucleotides (on average) incorporated into a primer strand.

MATERIALS AND METHODS

Materials

All nucleotides (dAMP, dTMP, dGMP, dCMP), Dowex-50W ion exchange resin (hydrogen form), and all organic reagents were purchased from Sigma Aldrich. Dimethylformamide (DMF) was purchased from Acros Organics. Klenow fragment DNA Polymerase exo-, KlenTaq DNA Polymerase, Therminator™ DNA Polymerase, Vent® exo- DNA Polymerase, AMV Reverse Transcriptase, and dNTPs were purchased from NEB. Platinum Taq High-Fidelity
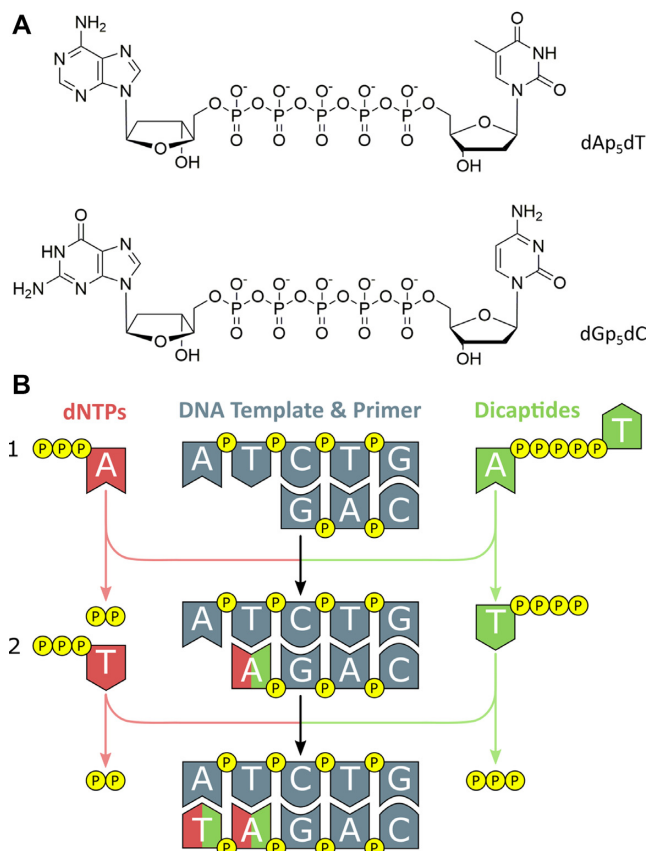

Figure 1. (**A**) Structure of the two pseudo-complementary dicaptides: dAp$_5$dT and dGp$_5$dC. Each is a nucleotide dimer containing a pentaphosphate bridge linking the two nucleoside monomers. (**B**) Scheme comparing polymerase extension of a DNA primer hybridized to a template using dicaptides versus using dNTPs. A single dicaptide molecule (here dAp$_5$dT) can be used for two base incorporations, yielding triphosphate as the final byproduct. By comparison, two canonical dNTPs (dATP and dTTP) are needed to accomplish the same extension, and two pyrophosphate molecules are generated as byproducts.

DNA Polymerase and Maxima H Minus Reverse Transcriptase were purchased from Thermo Fisher Scientific. All buffers used for enzymatic experiments were provided with the enzyme. All custom oligonucleotides were purchased from IDT. Plasmids were purchased from Addgene. High-performance liquid chromatography (HPLC) was performed using a system comprised of two Shimadzu LC-10AD pumps, SCL-10A controller, and SPD-M10A photodiode array detector. Waters SunFire® C18 5 μm 4.6 × 150 mm column was used for HPLC analysis. The thermal cycler used for PCR was Eppendorf Mastercycler Gradient. PAGE analysis was visualized using a Typhoon 9410 Variable Mode Imager.

Synthesis of dicaptides

The synthetic procedure for dicaptides (Supplementary Scheme S1) was adapted from a previous study where dinucleoside pentaphosphates were synthesized starting from trimetaphosphate (20). To a solution of trimetaphosphate tetrabutylammonium salt (195 mg, 0.21 mmol, 1 eq) in dry DMF (2.5 ml), 1-methylimidazole (55 mg, 54 μl, 0.68 mmol,

3.2 eq) and 2-mesitylene chloride (40 mg, 0.18 mmol, 0.86 eq) were added. The mixture was stirred at room temperature for 25 min, then added dropwise to a cooled flask containing a tetrabutylammonium salt of dAMP (0.135 mmol, 0.64 eq) or dGMP (0.135 mmol, 0.64 eq) in dry DMF (1.5 ml) while stirring over a period of 30 s. The ice-bath was then removed, and the reaction mixture was stirred at room temperature for 3 h. A solution of a tetrabutylammonium salt of dTMP (0.27 mmol, 1.3 eq) or dCMP (0.27 mmol, 1.3 eq) in dry DMF (3 ml) was added dropwise to the reaction mixture followed by the addition of anhydrous magnesium chloride (14 mg, 0.15 mmol, 0.7 eq). The mixture was then stirred at room temperature for 72 h. Afterward, the solution was cooled to 0°C (ice bath) and quenched by the addition of 100 mM triethylammonium acetate buffer (pH 7, 6 ml). The resulting solution was washed with chloroform (3 × 10 ml), then purified by RP-HPLC using a semipreparative C18 column. After pooling fractions and lyophilization, dAp_5dT and dGp_5dC were each obtained in 45% yield as white powders.

Steady-state kinetics measurements

Steady-state kinetics assays were performed following previously published procedures (21). The 13 nt primer (5′-CTAGGATCATAGC-3′) was end-labeled with T4 polynucleotide kinase and [γ-32P]-ATP following the manufacturer's protocol. The 20 nt DNA templates (5′-ATGGCGNGCTATGATCCTAG-3′, N = A, T or 5′-ATGGCGNTCTATGATCCTAG-3′, N = C, G) were annealed with $5′-^{32}P$-labeled 13 nt primer as described above. The annealed primer-template duplex (0.05 μM) was incubated with Klenow fragment exo- polymerase at 37°C for 5 min in the presence of individual dNTPs or dicaptides at varied concentrations. The parameters were adjusted to result in extents of reaction of 20% or less to maintain initial velocity conditions. The reaction was terminated with an equal volume of formamide gel loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol and bromophenol blue). Extension products were separated on 20% denaturing polyacrylamide gels containing 8 M urea. Gel band intensities of the primer and its extension products were quantified using a Typhoon 9410 Variable Mode Imager. Quantitative imaging of bands was carried out using ImageJ software to determine the fraction of primer extension. The velocity was plotted as a function of dNTP (or dicaptides) concentration and fit with the Michaelis−Menten equation to obtain the kinetic parameters, V_{max} and K_m. Reactions were performed three times and the mean (± standard deviation) of V_{max} and K_m are reported. The k_{cat} values were calculated by dividing V_{max} by the concentration of polymerase used. The efficiency of nucleotide incorporation was calculated by the ratio k_{cat}/K_m.

HPLC assay of stability

All nucleotides (dATP, dTTP, dAp_5dT, dGp_5dC) were incubated at 37°C at 200 μM nucleotide concentration in a commercial polymerase reaction buffer (NEBuffer 2). Buffer conditions at 1× were 50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT (pH 7.9 at 25°C), per the manufacturer. 200 μl aliquots were taken from each tube each week

an analyzed by HPLC. HPLC methods differed among analytes in order to resolve degraded compounds; the methods used are as follows. dATP: Isocratic flow of 4% acetonitrile and 96% 50 mM triethaylammonium bicarbonate buffer for 20 min. Flow rate: 1.00 ml/min. dTTP: isocratic flow of 3% acetonitrile and 97% 50 mM triethylammonium bicarbonate buffer for 20 min. Flow rate: 1.25 ml/min. dAp_5dT and dGp_5dC: isocratic flow of 5% acetonitrile and 95% 50 mM triethaylammonium bicarbonate buffer for 5 min, followed by a gradient increase in acetonitrile to 10% over 21 min. Flow rate: 0.50 ml/min.

Primer extension experiments

Reactions included 40 nt DNA templates, all of which shared a conserved region (20 nt starting from the 3′ end) complementary to a 20 nt 5′-Cy5-labeled primer. All primer extension reactions included 15 nM template and 10 nM primer, 10 μM dicaptides or dNTPs, DNA polymerase or reverse transcriptase, the appropriate reaction buffer, and DNase-free water up to 10 μl. Reactions were incubated at the appropriate temperature in accordance with the manufacturer's suggestions. After allowing enough time for extension, samples were dried using a Labconco centrifugal evaporator and suspended in 10 μl 0.1% Orange-G dye with 7 M urea in water. Samples were loaded onto a 20% polyacrylamide gel and electrophorized for 1.5 h at 20 A before visualizing using a Typhoon 9410 Variable Mode Imager.

Short (87 nt) PCR Amplicons

10 ng single-stranded 87 nt DNA excerpt of the human beta-globin gene (custom-ordered from IDT) was used as the amplification template. 0.5 μM forward and reverse primers, 200 μM dicaptides or dNTPs, and 2 units of Vent® exo- DNA polymerase were added along with the appropriate reaction buffer supplied by the manufacturer (NEB). For sequencing, the forward primer had an additional 50 nt of randomized sequence appended to its 5′ end. Reaction volumes were 50 μl. The thermal cycling method started with 30 s of incubation at 94°C, followed by 25 cycles of 15 s at 94°C (denaturation), 15 s at 58°C (annealing), and 1 min at 72°C (extension). Finally, tubes were incubated at 72°C for 5 min for primer extension to reach completion. Amplicons were purified using GeneJET PCR Purification Kit from Thermo Fisher Scientific. The purified amplicons were analyzed by loading onto a 2.5% agarose gel containing SYBR Gold dye. The gel was run in 1× TBE buffer for 1 h, after which it was visualized on a UV illuminator and captured using a smart phone camera. For sequencing data, amplicons containing an additional 50 nt of randomized sequence at the 5′ end were submitted to Elim Biopharm for Sanger sequencing.

Long (≥500 bp) PCR Amplicons

All nucleotides (dicaptides or dNTPs) were used at a concentration of 200 μM. The PCR was performed using a plasmid containing a wild-type *BRAF* insert (a gift from Dustin Maly, Addgene plasmid #40775). The PCR amplification of 500 bp fragment consisted of 98°C for 30 s 36
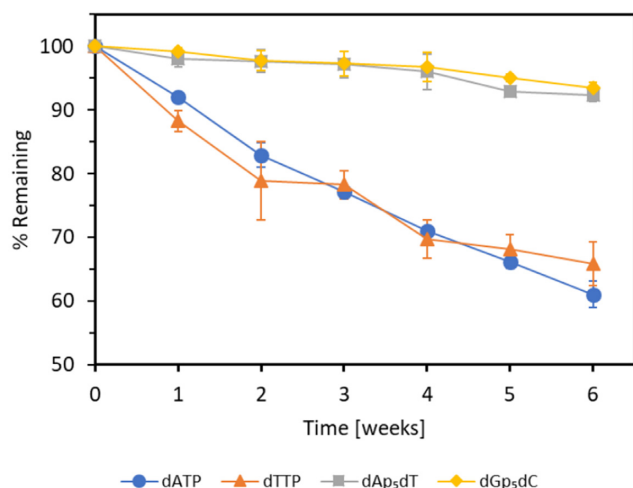
Figure 2. Enhanced hydrolytic stability of dicaptides compared with canonical dNTPs. dATP, dTTP, dAp$_5$dT and dGp$_5$dC were each incubated at 37°C in polymerase reaction buffer (NEBuffer 2, pH ≈ 7.6 at 37°C). At 6 weeks, the percent of starting material remaining for each compound was as follows: 61.0% ± 2.1% (dATP), 65.9% ± 3.4% (dTTP), 92.4% ± 1.1% (dAp$_5$dT), 93.5% ± 0.9% (dGp$_5$dC). Experiments were performed in triplicate; data are plotted as mean ± standard deviation.

cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 5 min, and a final extension at 72°C for 5 min. The PCR amplification of 2.1 kb fragment consisted of 98°C 30 s, 36 cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 5 min or 10 min, and a final extension at 72°C for 5 min. PCR products were analyzed by gel electrophoresis on a 1% agarose gel containing SYBR Gold dye. The gel was run in 1× TBE buffer at 75 V for 1 h, after which it was visualized on a UV illuminator and captured using a smart phone camera.

RESULTS

Synthesis and stability of dicaptides

There are six possible non-symmetric dicaptides; here we chose to study the pseudo-complementary variants (A+T and G+C) in order to balance nucleobase stoichiometry during double-stranded DNA synthesis reactions. The dimers were prepared by coupling activated trimetaphosphate with dAMP or dGMP followed by dTTP or dCTP, respectively. Dicaptides were purified by reverse-phase HPLC and characterized by NMR and mass spectrometry (see the Supplemental Data for details of synthesis and characterization). To compare the thermal stability of dicaptides with that of dNTPs under normal reaction conditions, nucleotides were incubated at 37°C in polymerase reaction buffer (Figure 2). Decomposition by hydrolysis was monitored by reverse-phase HPLC over the course of 6 weeks. Under these conditions, dicaptides exhibited superior hydrolytic stability to dNTPs, with an average of 93% of starting material remaining for dicaptides at six weeks, compared to only 63% on average for dNTPs after six weeks. After one week of incubating native dATP, a peak associated with the corresponding nucleoside diphosphate (dADP) was observed, confirming reactivity of the terminal phosphate. On the other hand, no substantial products of hydrol-

Table 1. Steady-state DNA polymerase (Klenow fragment exo-) efficiency with dicaptides versus dNTPs

Nucleotide	k_{cat}[min^{-1}]	K_m[μM]	k_{cat}/K_m[μM^{-1} min^{-1}]
dATP	8.6 ± 0.3	0.35 ± 0.05	25
d<u>A</u>p$_5$dT	3.4 ± 0.2	1.4 ± 0.2	2.4
dTTP	8.7 ± 0.3	0.24 ± 0.06	36
dAp$_5$d<u>T</u>	3.7 ± 0.2	2.4 ± 0.6	1.5
dGTP	15.4 ± 0.5	0.11 ± 0.01	140
d<u>G</u>p$_5$dC	9.7 ± 0.5	5.0 ± 0.6	1.9
dCTP	14.0 ± 0.4	0.07 ± 0.01	200
dGp$_5$d<u>C</u>	15.2 ± 0.1	2.6 ± 0.1	5.8

For dicaptides, incorporation of the underlined base was characterized in the experiment.

ysis were detectable for dicaptides until five weeks of incubation had elapsed. Dicaptide hydrolysis products appeared to be mixtures of nucleoside mono- and poly-phosphates.

Polymerase substrate capability of dicaptides

We then sought to quantify the efficiency of dicaptides as substrates for DNA polymerase. Comparing the steady-state kinetics of the two dicaptides with the four canonical dNTPs using the Klenow fragment of DNA Polymerase I (exo-), dicaptides are slightly less efficient than their native counterparts (Table 1). The average K_m value for dicaptides was 2.9 μM, about 15 times greater than that of dNTPs. However, k_{cat} values for dicaptides (8.0 min^{-1}) were comparable to those of dNTPs (11.7 min^{-1}). These results indicate that although somewhat higher concentrations are needed to attain peak reaction rates for dicaptides, maximal velocities for polymerase extension are nearly as high as those of native dNTPs. For most applications involving primer extension and PCR, a vast excess of nucleotides is almost always used, rendering the higher K_m value for dicaptides largely inconsequential.

Dicaptides in 4-base DNA synthesis

Screening of several DNA polymerases and reverse transcriptases revealed that Therminator™ DNA polymerase was yet more efficient at incorporating dicaptides than Klenow exo- enzyme (Figure 3A). Further primer extension experiments using this enzyme were performed in order to characterize dicaptide performance with various simple DNA templates (Figure 3B). Extension on a 40 nucleotide (nt) template containing an equal number of A and T bases was successful using only one dicaptide, dAp$_5$dT. Similarly, a template containing G and C bases was extended using only dGp$_5$dC. Using both dicaptides together, four-letter extension was accomplished on a mixed template. Additionally, time course experiments with the Therminator™ enzyme revealed a comparable degree of primer extension when using dicaptides *vs.* dNTPs at various time points (Supplementary Figure S1).

Testing dicaptides for PCR

As noted previously, one of the issues with employing dNTPs in PCR is that the byproduct pyrophosphate ac-
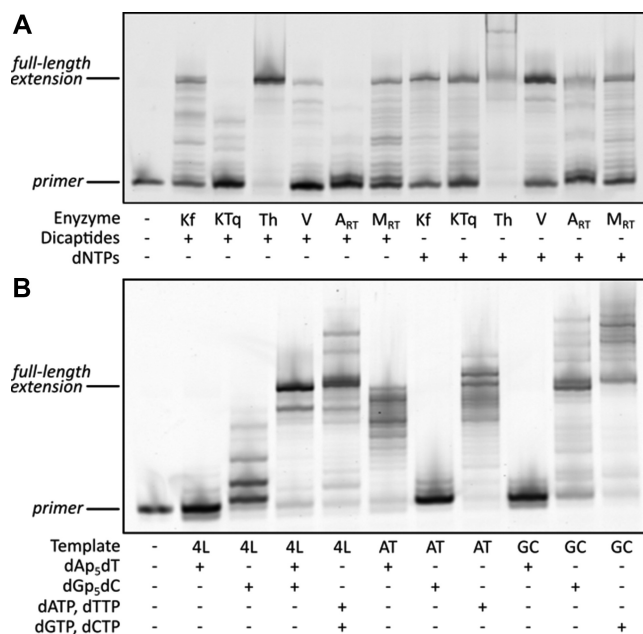
Figure 3. (**A**) Screening of several DNA polymerases and reverse transcriptases to identify an enzyme with high efficiency using dicaptides as substrates for DNA synthesis. Kf: Klenow fragment exo-; KTq: KlenTaq; Th: Terminator™; V: Vent® exo-; A_{RT}: AMV reverse transcriptase; M_{RT}: Maxima H- reverse transcriptase. Reactions contained 100 nM 20 nt 5′-Cy5-labeled primer, 150 nM 40 nt 4-letter template, 10 μM dicaptides or dNTPs (all four). Each reaction was incubated for 1 h at 37°C (Kf, A_{RT}), 55°C (M_{RT}), or 75°C (KTq, Th, V) with appropriate reaction buffers supplied by the manufacturer. Primer extension was comparable between dicaptides and dNTPs for Kf, Th, and M_{RT}, while KTq, V, and A_{RT} displayed a noticeably lesser degree of extension with dicaptides. The best performance with dicaptides was observed with Terminator™. (**B**) Characterization of dicaptide performance with simple oligonucleotide templates and the Terminator™ enzyme. All 40 nt templates contained a 20 nt region complementary to the 20 nt 5′-Cy5-labeled primer. The remaining 20 nt was comprised of all four letters (4L), exclusively A and T (AT), or exclusively G and C (GC). Reactions were incubated for 45 min at 75°C and contained 100 nM 20 nt 5′-Cy5-labeled primer, 150 nM 40 nt 4-letter template, 10 μM dicaptides or dNTPs. As observed with this experiment, dAp_5dT functions as a combination of dATP and dTTP, while dGp_5dT works as a combination of dGTP and dTTP. Employing two dicaptides in tandem allows for 4-letter primer extension.

cumulates over time and can inhibit primer extension. The inhibitory effect of triphosphate, the final byproduct of dicaptide-based DNA synthesis, was characterized and compared to that of pyrophosphate (Supplementary Figure S2). Even at relatively low concentrations, pyrophosphate completely inhibited polymerase extension. As pyrophosphate concentration increased, the extent to which pyrophosphorolysis occurred also increased. In contrast, triphosphate was found to be far less inhibitory than pyrophosphate and did not appear to cause triphosphorolysis to any measurable degree.

Next, PCR experiments tested the potential of dicaptides in DNA amplification. Vent® exo- DNA polymerase was identified as a viable thermostable enzyme to process dicaptides while thermocycling (Supplementary Figure S3). Amplification of a single-stranded 87 nt excerpt from the human beta-globin (*HBB*) gene resulted in a single band of the expected size when analyzed by gel electrophoresis (Figure
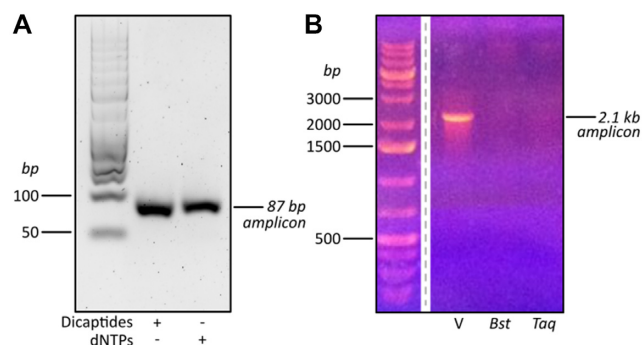

Figure 4. (**A**) PCR amplification of an 87 nt segment of the human beta-globin gene using dicaptides. After running samples on a 2.5% agarose gel, a single clear band of the expected size (87 bp) was apparent. This band matches that which was produced using dNTPs under otherwise identical conditions. (**B**) 2.1 kb amplicons of a plasmid containing the *BRAF* gene were produced via PCR using dicaptides in place of dNTPs. Although PCR with Bst or Taq polymerases did not produce any observable amplicon, a single band of the expected size (2.1 kb) was evident using Vent® exo-DNA polymerase (V) and extension times of 10 minutes per cycle.

4A). Subsequent Sanger sequencing confirmed that the sequence of the amplicon matched that of the target, demonstrating that canonical pairing rules are in effect for dicaptides as expected (Supplementary Figure S4). Encouraged by this, we further tested PCR of longer amplicons, with appropriately increased extension times due to the elongated templates. Experiments amplifying DNA corresponding to the *BRAF* gene confirmed that an amplicon of 500 bp was efficiently produced using the Vent® enzyme with the two dicaptides, and a 2.1 kilobase (kb) segment from the same gene was successfully amplified when extension times were increased to 10 min (Figure 4B and Supplementary Figure S5). Finally, we tested whether the enhanced stability and lack of pyrophosphate might provide an advantage for dicaptides in extended PCR amplification protocols, which commonly require both long extension times and a high number of thermal cycles. Using dicaptides, 500 bp *BRAF* amplicons could be detected by gel electrophoresis starting with as little as 100 pg of BRAF plasmid (Figure 5B). Under similar conditions with dNTPs, amplicons could not be detected when starting with any amount <10 ng of plasmid (Figure 5A). This demonstrates a ca. 100-fold increase in sensitivity using dicaptides versus dNTPs under these conditions.

DISCUSSION

Our data show that the dimeric nucleotides studied here constitute a novel nucleotide design that addresses two issues that limit dNTPs: namely, that they are prone to hydrolysis and yield pyrophosphate when incorporated into DNA by polymerase. Dicaptides are shown to be much more stable to hydrolysis than canonical dNTPs are, presumably because the terminal phosphate is blocked by substitution with another nucleotide. Prior studies have shown that alkyl substitution of the terminal phosphate of a dNTP enhances stability, a finding with which the current results are in accordance [17]. It seems likely that the added stability at pH values near neutral is the result of the fact that
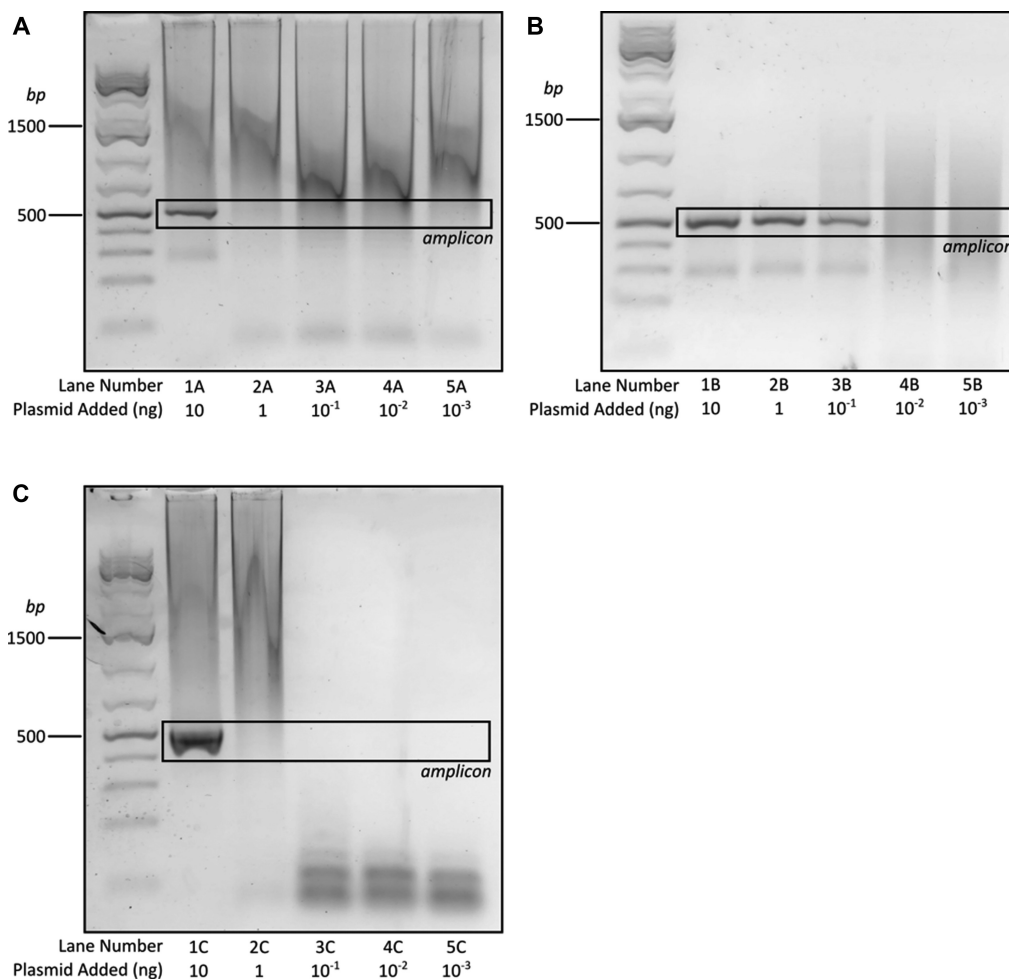
Figure 5. Limit of detection of *BRAF* plasmid DNA (500 bp amplicon) using PCR under extended conditions (45 cycles, 5-minute extension). Each thermal cycle included a 15-second denaturing step at 94°C, 30-second annealing step at 60°C, and a 5-minute extension step at 72°C. Amount of starting material (*BRAF* plasmid) is shown. (**A**) Using normal dNTPs, 10 ng of plasmid was the lowest that could be detected by gel electrophoresis using dNTPs (lane 1A), indicated by the presence of a 500 bp amplicon band. (**B**) Using dicaptides, 100 pg of plasmid was detected (lane 3B), demonstrating a 100-fold increase in sensitivity. C) Control experiment showing limit of detection of *BRAF* plasmid with PCR under normal conditions (36 cycles) using dNTPs. Each cycle was the same as described above, except that the extension step was 30 seconds instead of 5 minutes. The limit of detection was also 10 ng of plasmid DNA under these conditions.

all phosphates in dicaptides are doubly substituted, altering the phosphate pK_a markedly. A phosphate monoester has one basic oxygen with pK_a of ∼5, while a phosphate diester has a pK_a near ∼1 (22). As a result, the former compounds can be protonated much more readily near neutral pH, potentially enabling proton transfer from the terminal phosphate to stabilize the leaving diphosphate. In contrast, dicaptides are not expected to be protonated to any significant extent, and thus in some respects resemble phosphodiesters, which are quite stable. Notably, dicaptides' increased stability to hydrolysis also allows both for more reliable long-term storage and improved PCR performance under extended conditions. Dicaptides were successfully employed here with Vent® exo- polymerase in PCR to amplify multiple DNA targets. Although for short, high-abundance targets the dicaptides show no benefit over canonical dNTPs, better amplification efficiency is seen for the new dimeric nucleotides under conditions of longer extension times and higher cycle numbers, resulting in as

much as 10^2-fold higher sensitivity of target detection. The increased PCR efficiency of dicaptides relative to dNTPs under these conditions appears to be a result of the stability of dicaptides, in contrast to dNTP depletion due to thermal hydrolysis.

Of the enzymes tested here, the Therminator™ polymerase was shown to have optimal performance using dicaptides as substrates under isothermal conditions. The reason for their lowered efficiency relative to dNTPs is not yet clear; examination of known polymerase structures suggest that there is ample room for the 'extra' phosphate and nucleotide at the end of the chain, outside the enzyme pyrophosphate channel. Moreover, nucleoside tetra- and penta-phosphates are very good polymerase substrates (15). It seems possible that the appended terminal nucleotide of dicaptides may compete for active site binding; more detailed kinetics studies will likely be helpful in understanding this behaviour. It also remains to be seen if other en-

zymes can be identified or evolved that accept the dimeric nucleotides with yet higher efficiency.

Our data comparing pyrophosphate to triphosphate confirm that the final byproduct of dicaptide-mediated DNA synthesis, triphosphate, is less inhibitory towards primer extension as compared with pyrophosphate. The origin of pyrophosphate-mediated polymerase inhibition is pyrophosphorolysis, in which pyrophosphate attacks the terminal internucleotide phosphate (catalyzed by the enzyme) on the primer strand of the template-primer duplex (12), reversing DNA synthesis and shortening the primer. Given that nucleoside tetraphosphates are efficient polymerase substrates, it is not yet clear why triphosphate is less inhibitory. We speculate that one possible reason for this might be that the 'triphosphorolysis' reaction is more energetically uphill, due to greater charge-charge repulsion. Conversely, this would explain why nucleoside tetraphosphates are better polymerase substrates than the canonical triphosphates; the tetraphosphates would release more energy upon expulsion of triphosphate, and by the Hammond postulate, exhibit a lower barrier to reaction.

Our primer extension studies unmasked the potential of dicaptides to be used as a combination of two nucleotides simultaneously. There are eight possible pentaphosphate-bridged dicaptides of the four canonical deoxyribonucleosides: the symmetrical cases (dAp_5dA, dCp_5dC, dGp_5dG, dTp_5dT), the pyrimidine and purine heterodimers (dCp_5dT and dAp_5dG), the non-complementary heterodimers (dAp_5dC and dGp_5dT) and the pseudo-complementary cases studied here (dAp_5dT and dGp_5dC). The current results document the ability of the two pseudo-complementary dicaptides to act as substitutes of all four canonical dNTPs; the tethering of complementary nucleotides guarantees equal concentrations of nucleotides during double-stranded DNA synthesis such as occurs in PCR. For example, in copying a stretch of poly-A, the thymidine end of the dAp_5dT dicaptide is consumed, releasing increasingly high concentrations of p_4dA, which would then be consumed in equal amounts during the complementary strand synthesis. Thus the concentrations and stoichiometries of the nucleotides are self-adjusting regardless of imbalance of the sequence being amplified. It will be of interest in future studies to delineate the properties and applications of the other dicaptides, which may present some of their own benefits in different applications.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

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